

# Identification and Optimization of a Novel HIV-1 Integrase Inhibitor

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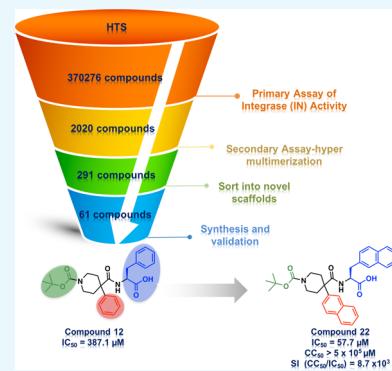
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**ABSTRACT:** Human immunodeficiency virus-1 (HIV-1) is the causative agent of acquired immunodeficiency syndrome (AIDS). HIV-1, like all retroviruses, stably integrates its vDNA copy into host chromatin, a process allowing for permanent infection. This essential step for HIV-1 replication is catalyzed by viral integrase (IN) and aided by cellular protein LEDGF/p75. In addition, IN is also crucial for proper virion maturation as it interacts with the viral RNA genome to ensure encapsulation of ribonucleoprotein complexes within the protective capsid core. These key functions make IN an attractive target for the development of inhibitors with various mechanisms of action. We conducted a high-throughput screen (HTS) of ~370,000 compounds using a homogeneous time-resolved fluorescence-based assay capable of capturing diverse inhibitors targeting multifunctional IN. Our approach revealed chemical scaffolds containing diketo acid moieties similar to IN strand transfer inhibitors (INSTIs) as well as novel compounds distinct from all current IN inhibitors including INSTIs and allosteric integrase inhibitors (ALLINIs). Specifically, our HTS resulted in the discovery of compound 12, with a novel IN inhibitor scaffold amenable for chemical modification. Its more potent derivative 14e similarly inhibited catalytic activities of WT and mutant INs containing archetypical INSTI- and ALLINI-derived resistant substitutions. Further SAR-based optimization resulted in compound 22 with an antiviral EC<sub>50</sub> of ~58 μM and a selectivity index of >8500. Thus, our studies identified a novel small-molecule scaffold for inhibiting HIV-1 IN, which provides a promising platform for future development of potent antiviral agents to complement current HIV-1 therapies.



## INTRODUCTION

An essential replication step for all retroviruses, including HIV-1, is the stable integration of viral DNA (vDNA) into the host genome for a productive infection. This process requires the formation of the preintegration complex (PIC), a key intracellular structure that contains cDNA in complex with the viral integrase (IN) protein (intasome).<sup>1,2</sup> The HIV-1 intasome interacts with the transcription co-activator LEDGF/p75,<sup>3,4</sup> which tethers PICs to select sites on chromatin during integration.<sup>5–7</sup> Oligomeric IN catalyzes two sequential reactions for insertion of linear viral cDNA into cellular chromatin.<sup>8</sup> Initially, IN removes a GT dinucleotide from the 3' terminus of each viral DNA end (3' processing) and, subsequently, catalyzes concerted transesterification reactions (strand transfer) to integrate recessed viral DNA ends into the host genome. In addition, IN has a second crucial role in HIV-1 replication. Specifically, IN interacts directly with the viral RNA genome during virion morphogenesis.<sup>9–11</sup> This allows for correct viral maturation and the incorporation of ribonucleoprotein complexes into the capsid shell for subsequent infection of target cells. Essential roles of IN in viral integration and virion maturation present therapeutic routes for targeting HIV-1.

IN strand transfer inhibitors (INSTIs) are integral components of antiretroviral regimens to treat people living

with HIV-1. Clinically relevant INSTIs include raltegravir (RAL), elvitegravir (EVG), dolutegravir (DTG), bictegravir, and cabotegravir (BIC).<sup>12–14</sup> HIV-1 resistance to INSTIs has already emerged in patients. The therapeutic use of the first-generation RAL and EVG resulted in the evolution of drug-resistant IN substitutions within the active site,<sup>15,16</sup> whereas second-generation DTG and BIC display a higher barrier of resistance. Their use, however, has resulted in IN substitutions both within the active site and at spatially distinct sites.<sup>17–21</sup> Due to the continued evolution of viral resistance, research is ongoing into improved INSTIs.<sup>22–24</sup>

In addition to these active site inhibitors, the development of a second class of IN inhibitors has more recently generated significant interest. These inhibitors, termed allosteric IN inhibitors [ALLINIs, also referred to as noncatalytic site integrase inhibitors (NCINIs); LEDGINs or INLAIs], inhibit HIV-1 IN interactions with LEDGF/p75 and promote inactive hyper-(or aberrant) higher-order IN oligomerization.<sup>9,25–30</sup>

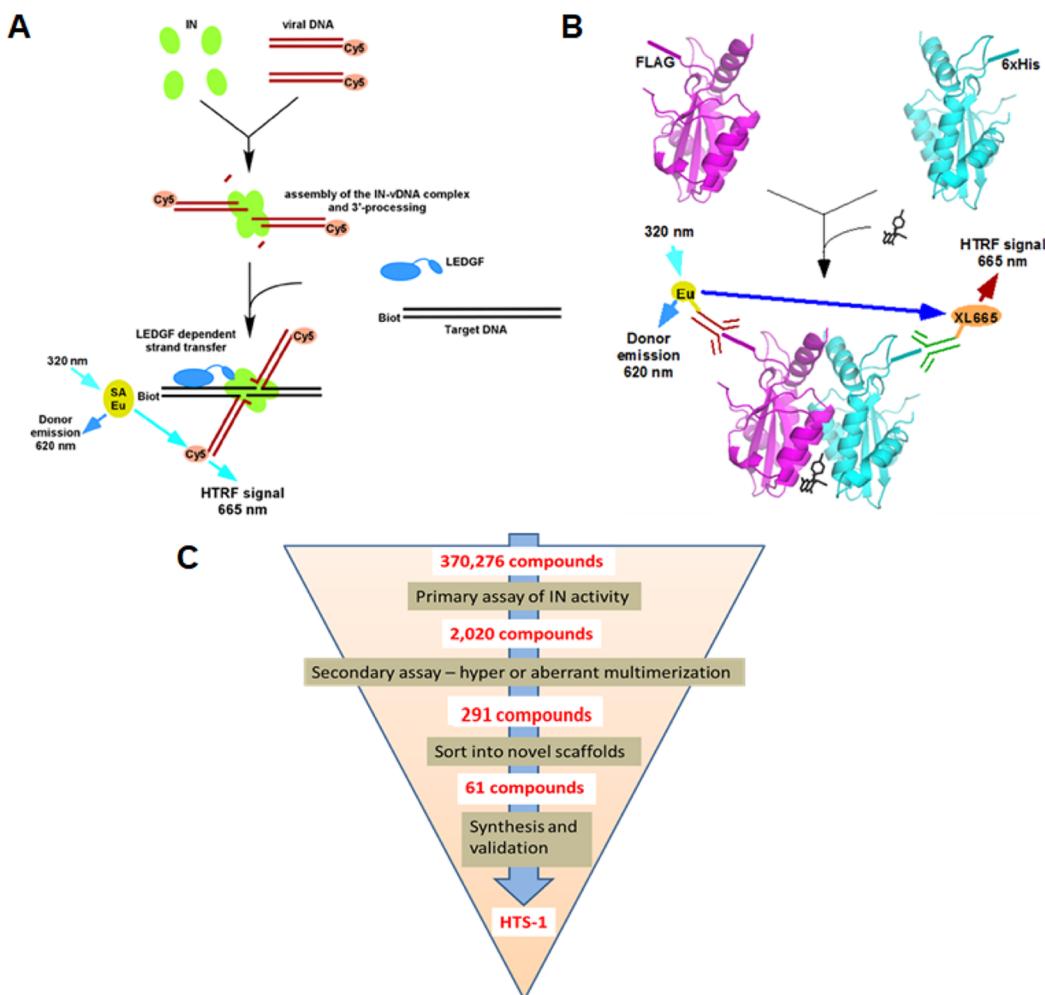
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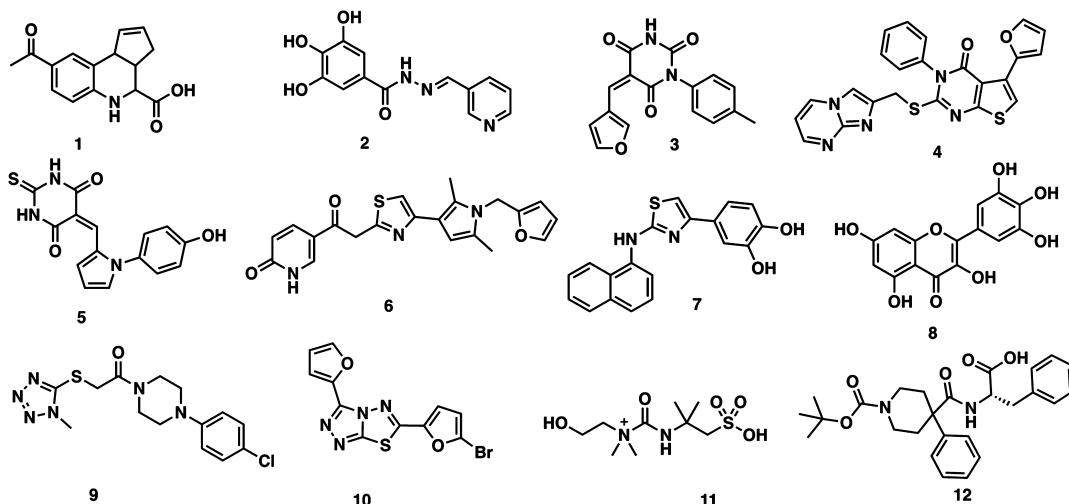


**Figure 1.** (A) Schematic of LEDGF/p75-dependent HTRF based assay. (B) Schematic of the IN multimerization HTRF-based assay. Shown are the IN proteins with the corresponding tags and representative ALLINI. (C) Graphical representation of HTSs and resulting compounds with hit numbers.

ALLINIs bind within the LEDGF/p75 binding pocket located on the IN catalytic core domain (CCD) dimer interface.<sup>31,32</sup> Inhibitor binding blocks LEDGF/p75 interaction with HIV-1 intasomes during the early stage of infection decreasing integration efficiency and altering LEDGF/p75 guided target site selection.<sup>33–35</sup> This alteration in HIV-1 integration by ALLINIs has been reported to retarget integration toward repressive chromatin regions, thus leading to increased latency and decreased reactivation of proviruses.<sup>36–38</sup> However, their primary mechanism of action is during the late stage of the viral lifecycle through the promotion of hyper IN multimerization which affects assembly during virion maturation in a LEDGF/p75-independent manner.<sup>25,31,32,34,39,40</sup> It is likely that these higher-order IN oligomers are unable to effectively bind the viral RNA genome, resulting in the formation of eccentric noninfectious virions with the ribonucleoprotein complexes (RNPs) mislocalized outside of the capsid shell.<sup>9</sup> It is still unclear if there are other effects of ALLINI treatment during virion assembly. Multiple ALLINI scaffolds have been examined with a focus on those with a quinoline or a pyridine-based central moiety and other scaffolds containing isoquinoline, pyrimidine, thiophene, and indole cores.<sup>20,31,32,41–45</sup> ALLINI resistance has been shown to rapidly arise in cell culture with the archetypical ALLINI-derived resistant

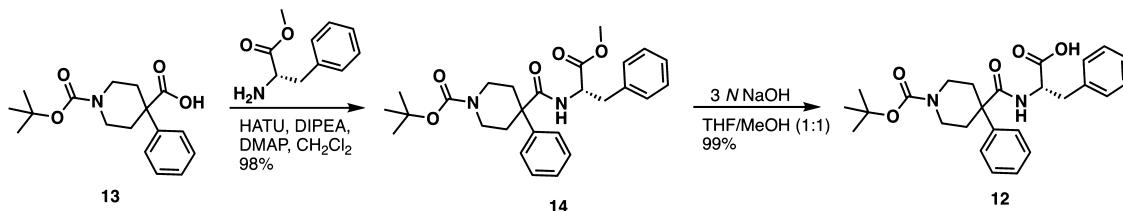
mutations being within the LEDGF/p75-inhibitor binding pocket.<sup>31</sup> Improved ALLINIs have been shown to have significantly enhanced selective pressure against the evolution of resistance due to the fact that substitutions are needed not only in the inhibitor-binding site but also in distal areas of the protein.<sup>43,46</sup>

To identify novel inhibitors of HIV-1 IN with distinct structures from INSTIs and ALLINIs, we conducted a high-throughput screen (HTS) of ~370,000 compounds. This HTS utilized a homogeneous time-resolved fluorescence (HTRF)-based assay capable of capturing different types of IN inhibitors including those that impair functional multimerization of IN, interfere with IN binding to its cellular cofactor LEDGF/p75, or inhibit its catalytic activities, all in a single assay. These studies resulted in the identification of compound **12** with a distinct scaffold amenable to chemical modification, albeit with relatively weak potency ( $IC_{50}$  of 387  $\mu$ M). Importantly, initial optimized compound **14e** was able to overcome both archetypical INSTI- and ALLINI-derived resistance mutations *in vitro*. For these reasons, we have chosen to extend our optimization efforts. SAR-based optimization resulted in the selection of compound **22** with an antiviral  $IC_{50}$  of ~58  $\mu$ M and a selectivity index of >8500.



**Figure 2.** Representative hits obtained from the HTS campaign.

**Scheme 1. Synthetic Route for the Generation of 12**



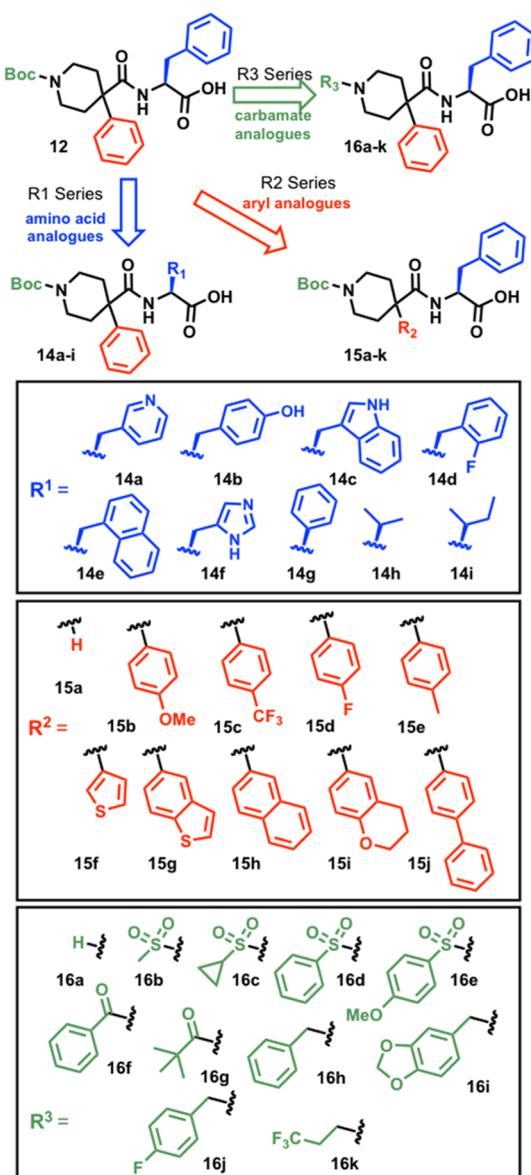
## RESULTS

**Identification of Compounds Targeting HIV-1 Integrase.** An HTS of ~370,000 compounds was performed at The Scripps Research Institute Molecular Screening Center to identify inhibitors with broad activity against HIV-1 IN (Figure 1, also see the Supporting Information Methods for detailed methods and setup). Efficient and rapid screening was accomplished with an HTRF-based assay that measured viral DNA integration into the target DNA catalyzed by IN in the presence of LEDGF/p75, termed the “LEDGF/p75-dependent integration assay”. The addition of LEDGF/p75 resulted in a >6-fold increase in the total HTRF signal over similar reaction conditions without the cellular cofactor, mirroring cell-based LEDGF/p75 depletion studies showing a stimulatory role of the cellular cofactor in HIV-1 integration.<sup>47</sup> The resulting hit set from the LEDGF/p75-dependent integration assay was then further refined using a counterscreen to eliminate compounds that were fluorescent quenchers (see the Methods for PubChem BioAssay accession numbers and hit counts). The resulting hit set of 2020 compounds was then subjected to an IN multimerization screen which monitored the effect of the compounds on the interaction between two differentially tagged full-length wild-type HIV-1 IN proteins.<sup>48</sup> In this case, an HTRF signal was detected upon IN–IN interaction and compounds that promote hyper-multimerization increase fluorogenic output over normal lower-order IN oligomers. After counterscreening, the resulting multimerization-specific IN inhibitors (291 hits in total) were selected for hit-to-lead optimization experiments.

The 291 compounds possessed a wide range of chemical scaffolds and functionality, including flavonoids, various heterocyclic systems, unsaturated carbonyls, hydrazines, and thioureas (Figure S1). In addition, compounds containing

diketo acid moieties were also identified (compound 8), providing validation of the methodology (Figures 2 and S2).<sup>49</sup> These compounds were then evaluated by considering drug properties through screening for adherence to Lipinski's rule of 5, the presence of potential reactive functionality, structural diversity, and synthetic feasibility. Based on the application of these criteria, 61 compounds from the hit set were either purchased from chemical vendors or synthesized. Representative structures demonstrating the chemical diversity of this set are shown in Figure 2. Several of these compounds were identified as false positives due to fluorescence interference in the hit validation process, while others failed to demonstrate the desired activity in our hands.<sup>50</sup> For example, a commercial sample of compound 11 was found to show promising increased hyper-multimerization IN activity with an EC<sub>50</sub> of 2 μM, but this activity was not reproducible when this compound was subsequently synthesized. Compound 12, however, displayed more promising results. A commercial sample of the compound inhibited IN catalytic activity with an IC<sub>50</sub> value of 600 μM. The compound was then re-synthesized in two steps from the commercially available N-Boc-4-phenylpiperidine-4-carboxylic acid (13, Scheme 1). This process involved the straightforward coupling of the carboxylic acid moiety with L-phenylalanine methyl ester to give the ester and subsequent saponification to afford the desired compound. The synthetic sample similarly (IC<sub>50</sub> of 387 μM) inhibited IN catalytic activity. Having demonstrated reproducible activity, compound 12 was selected for subsequent hit-to-lead optimization.

**Hit Expansion Studies of Compound 12.** Analysis of the chemical structure of 12 shows that the molecule is composed of three basic structural motifs (colored in Figure 3) appended to an isonicotinic acid core. Preparation of a small library of



**Figure 3.** Analogues of HTS-derived lead compound 12 for the initial hit expansion studies.

analogues for hit expansion studies could be accomplished through modification of the synthesis of 12 shown in Scheme 1 (full library of generated compounds in Figure S2). Therefore, a library of 31 analogues (Figure 3) was designed using commercially available precursors to explore the systematic modification or removal of these functional groups. This first series of analogues (series 1) was tackled in order to increase complexity based on the number of synthetic transformations required. The most straightforward of these compounds focused on variation of the amino acid portion of 12. In total, nine analogues (14a–14i) were prepared through replacement of the phenylalanine portion of the molecule to explore the role of the amino acid side chain. The benzyl group present in phenylalanine was replaced with a variety of aromatic and heteroaromatic systems and two alkyl groups. The synthesis of these compounds was performed using the same reaction sequence, as shown in Scheme 1, with the corresponding commercially available methyl esters of selected amino acids as the coupling partners. The next group of

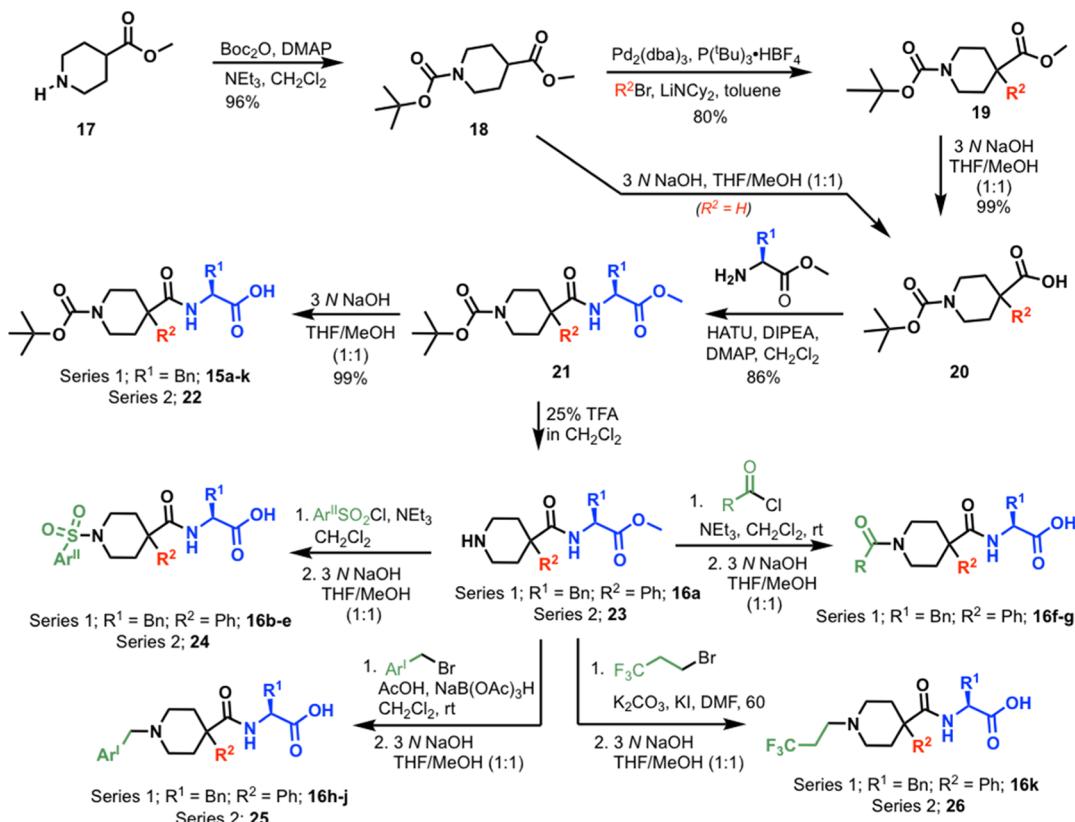
analogues in this series (compounds 15a–15j) involved variation of the aryl group at the 4-position of the piperidine ring in the parent compound. Starting with commercially available methyl isonipecotate (17, Scheme 2), compound 15a, which lacks the aryl ring, was prepared *via* a four-step process. This involved protection with a Boc group to give 18, subsequent saponification, and application of the same coupling and saponification sequence shown previously for the preparation of compound 12. Variation of the aryl groups at the 4-position of the piperidine ring in analogues 15b–15j was accomplished with Boc-protected isonipecotate 18 by employing a modified version of Hartwig's palladium-catalyzed  $\alpha$ -arylation protocol.<sup>51</sup> The resulting N-Boc-4-aryl ester 19 could once again be saponified to afford acid 20 and subsequently taken through the coupling sequence to install the phenylalanine group, providing 15b–j. The last group of analogues in Series 1 involved variation of the R<sup>3</sup> group on the piperidinyl nitrogen. Starting from the N-Boc aryl ester 21 (where R<sup>1</sup> = Bn and R<sup>2</sup> = Ph), the removal of the Boc group with 25% trifluoroacetic acid (TFA) in dichloromethane (CH<sub>2</sub>Cl<sub>2</sub>) gave compound 16a. From compound 16a, the various R<sup>3</sup> groups were installed through sulfonylation, acylation, reductive amination, and alkylation protocols. A final saponification reaction provided sulfonamides 16b–16e, amides 16f–16g, benzyl derivatives 16h–16j, and trifluoropropyl analogue 16k, respectively.

All the compounds synthesized as a part of this first series were tested for their ability to inhibit IN catalytic activity *in vitro*. Compounds were selected as “active” if they displayed improved potency compared to parent compound 12. We selected the LEDGF/p75-dependent integration assay for testing our compounds due to its ability to detect not only compounds that alter IN multimerization but other functions of IN including catalytic activity and LEDGF/p75 binding. This broad range of activity was desired as modifications to our parent compound could have unexpected pleiotropy effects. As shown in Table 1, seven out of the 31 compounds fit these criteria, with each of these compounds displaying a potency of at least twofold better than compound 12. Interestingly, analogues of each of the three subunits (R<sup>1</sup>, R<sup>2</sup>, and R<sup>3</sup>) appeared on this list. For the amino acid side chain, only one compound, the naphthyl-derived 14e, showed activity comparable to the parent phenylalanine-containing system. Among the 4-aryl derivatives, compounds 15d, 15h, and 15j all showed promising activity, although the biphenyl derivative 15j was 2–3-fold more active than the other two. Similarly, 16d, 16h, and 16k showed the best activity of the R3 analogues, with 16k being the most active of the group, albeit with lower IC compared to BIB-II.<sup>48</sup>

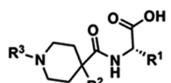
Compound 14e, which exhibited ~fourfold higher potency than 12, was tested further against archetypical ALLINI-derived resistant IN mutations A128T and H171T and the most common RAL-derived resistant mutant G140S/Q148H IN.<sup>48,52–55</sup> 14e exhibited a similar potency for WT and all three mutant INs (Table 2 and Figure S4). These results suggest that 14e targets HIV-1 IN through a novel interface as substitutions of residue keys for interacting with ALLINIs (His-171 and Ala-128) or INSTIs (Gly-140 and Gln-148) do not affect inhibitor potency making this series of compounds interesting for further development.

Next, we have synthesized another series of compounds by combining the functional groups in Table 1 to derive additional 19 compounds (22, 24a–f, 25a–f, and 26a–f,

**Scheme 2.** Synthetic Approach for the Preparation of the Initial Series of Analogs through Variation of the Amino Acid, Aryl, and Carbamate Portions of 12



**Table 1. Chemical Structure and Inhibition of HIV-1 IN Catalytic Activity *In Vitro* by Active Compounds from Series 1 Exhibiting Higher Potency than Parental 12**



Compd ID	R <sup>1</sup>	R <sup>2</sup>	R <sup>3</sup>	IC <sub>50</sub> (μM)
12				387.10±39.69
14e				90.32±14.00
15d				169.90±30.67
15i				157.50±80.20
15k				64.42±7.55
16d				189.29±8.16
16h				193.87±8.50
16k				73.33±2.35
BIB-II	-	-	-	0.12±0.04

**Table 2. Inhibitory Activity of 14e against WT and Drug-Resistant INs**

IN substitutions	IC <sub>50</sub> (μM)
WT	90.35 ± 4.72
A128T	86.82 ± 2.98
H171T	114.4 ± 9.79
G140S/Q148H	103.1 ± 3.12

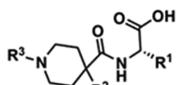
**Scheme 2.** These 19 compounds represent all the possible combinations of two R<sup>1</sup> groups (including the phenyl group of

12), three distinct R<sup>2</sup> substituents, and three R<sup>3</sup> groups. Upon examining inhibitory activities of this series (Table 3 and Figure S5), following conclusions could be drawn: (1) the two R<sup>1</sup> substituents did not provide dramatically different results in the LEDGF/p75-independent assay (e.g., 24a vs 24d; 25b vs 25e; and 26c vs 26f); (2) the effect of the R<sup>2</sup> substituent appears to be dependent upon the R<sup>3</sup> substitution; and (3) the benzenesulfonyl derivatives 24a–e showed the highest potency among all R<sup>3</sup> groups, while the trifluoromethyl derivatives were consistently less potent than expected based on the activity of 16k. Based on these results, two additional substituted benzenesulfonyl compounds, the 4-nitro derivative 27 and the 4-trifluoromethyl derivative 28, were also prepared. These compounds also showed very good potency, with 27 being the most potent of all the synthesized compounds exhibiting ~25-fold higher potency than compound 12.

**Evaluation of Cellular Inhibitory Potential and Cytotoxicity.** Two most active compounds 22 and 27 with *in vitro* IC<sub>50</sub> values of 44 and 16 μM, respectively, were selected for cell-based infectivity analysis (Table 3). 22 displayed an antiviral EC<sub>50</sub> of 58 μM with very limited cytotoxicity (CC<sub>50</sub> > 500 mM) (Table 4). While 27 exhibited a relatively higher antiviral potency (EC<sub>50</sub> of 17 μM), this compound was cytotoxic (CC<sub>50</sub> 60 μM). Accordingly, our results identify 22, which displays a selectivity index of >8500, as a promising candidate for future development of novel HIV-1 inhibitors.

## DISCUSSION

HTS campaigns performed by Merck and Boehringer Ingelheim (BI) have been highly successful, resulting in FDA-approved INSTIs and emerging ALLINIs. Merck

**Table 3. Chemical Structure and *In Vitro* IC<sub>50</sub> Values of Compounds from Series 2**

Compd ID	R <sup>1</sup>	R <sup>2</sup>	R <sup>3</sup>	IC <sub>50</sub> (μM)
22				45.91±5.0
24a				47.63±4.14
24b				87.46±4.00
24c				91.32±0.70
24d				55.58±3.27
24e				54.68±4.30
24f				150.19±9.30
25a				181.14±6.98
25b				353.43±54.50
25c				145.48±2.60
25d				>500
25e				>500
25f				124.82±33.40
26a				262.93±26.60
26b				337.80±2.94
26c				202.74±48.90
26d				93.53±0.01
26e				180.16±36.6
26f				206.34±6.20
27				16.96±1.98
28				55.99±2.51
BIB-II	-	-	-	0.12±0.04

**Table 4. Antiviral Activity, Cytotoxicity, and Selectivity Index of Optimized Compounds**

compd ID	inhibitory potential IC <sub>50</sub> (μM)	cytotoxicity CC <sub>50</sub> (μM)	selectivity index CC <sub>50</sub> /IC <sub>50</sub>
22	57.70 ± 6.18	>500,000	8665.5
27	16.96 ± 3.79	60.27 ± 17.3	3.6

employed preassembled IN viral DNA complexes to selectively monitor the strand transfer activity of IN and was instrumental in discovering INSTI.<sup>56,57</sup> A later HTS endeavor for inhibitors of 3'-processing was utilized by BI and led to the discovery of ALLINIs.<sup>58,59</sup> Our complementary HTS addresses two avenues missed by these previous screens: (i) the role of a key cellular cofactor LEDGF/p75 and (ii) directly targeting IN multimerization in the absence of viral DNA complexes.

Our HTS identified a wide range of chemical scaffolds and functionality, including flavonoids, various heterocyclic systems, unsaturated carbonyls, hydrazines, diketo acids, and thioureas, providing a range of scaffolds which could be of interest to medicinal chemists for future development of these hit compounds as potential HIV-1 IN inhibitors. In the present study, our efforts have been focused on **12** due to its distinct structure compared to known IN inhibitors including INSTIs and ALLINIs. Consistent with this observation, **14e**, an improved analogue of **12**, exhibited a similar potency with respect to WT and mutant INs containing the substitutions

that confer substantial resistance to INSITs and ALLINIs. Thus, the new series of compounds identified here could complement the known HIV-1 IN inhibitors. Future studies are warranted to elucidate structural and mechanistic bases of action of potent derivatives of **12**.

Our SAR-based medicinal chemistry efforts of **12** have highlighted the importance of all three subunits (R<sup>1</sup>, R<sup>2</sup>, and R<sup>3</sup>) in the overall antiviral potency. R<sup>1</sup> subunits displayed the best activity with either the parent phenylalanine **12** or naphthyl-derived **14e**. R<sup>2</sup> subunits of 4-aryl derivatives, compounds **15d** and **15h**, and biphenyl derivative **15j** appeared to be the most potent. Finally, R<sup>3</sup> subunits of sulfonamides **16d**, benzyl derivatives **16h**, and trifluoropropyl analogue **16k** showed the best activity (Table 1).

Subsequent combinatorial approaches identified compounds with improved potency (Table 3). Two of the most active compounds **22** and **27** inhibited IN catalytic activity *in vitro* with IC<sub>50</sub> values of 45 and 17 μM, respectively. Consistent with these results, **22** and **27** inhibited HIV-1 replication with EC<sub>50</sub> values of 58 and 17 μM, respectively. This close agreement between IC<sub>50</sub> and EC<sub>50</sub> strongly supports that these compounds are targeting HIV-1 IN in infected cells. While **22** was slightly less potent than **27**, the former exhibited an excellent selectivity index of >8500, whereas **27** was cytotoxic. Other studies investigating novel non-INSTI-based IN inhibitors have seen the development of chemical scaffolds such as pyrrolopyridine (41 pM), benzene (3.9 nM), 3-quinolineacetic acid (15 nM), a variety of modified pyridines (~4–20 nM), naphthyridine (70 nM), quinoline (630 nM), isoquinoline (1.1 μM), picolinamide (19 μM), and thiophene-carboxylic acid (103 μM).<sup>30,41,58,60–69</sup> These compounds displayed a range of therapeutic indexes with the best to date being pyrrolopyridine STP0404 reported to have >24,000 selectivity against HIV-1. Another interesting case was seen with the *t*-butylsulfonamide scaffold (39 nM) which was amenable to the development of a fluorescence polarization probe against HIV-1 IN.<sup>70</sup> The improvement of ALLINIs in the literature suggests that these newly discovered compounds reported here could see a similar trajectory for improvement while overcoming ALLINI-derived resistance mutations. Taken together, these findings highlight **22** for future development as a promising lead inhibitor of HIV-1 IN with the novel mechanism of action.

## METHODS

**Compound Synthesis.** Detailed methods are available in the Supporting Information methods.

**Recombinant Protein Expression and Purification.** 6XHis and Flag-tagged full-length HIV-1 IN and LEDGF/p75 were expressed in *Escherichia coli* BL21 (DE3) strain and purified as described previously.<sup>25,33,48,71,72</sup> HIV-1 IN domains were purified as previously described.<sup>25,73</sup>

**HTRF Assays.** IN catalytic activities were assayed in the presence and absence of LEDGF/p75 using HTRF-based approaches.<sup>41,44,48,55</sup> In brief, the assay monitors the integration product by measuring the intensity of a time-resolved FRET signal between two fluorophores: one on the donor DNA substrate (viral DNA) which is labeled at its 5' end with a Cy5 fluorophore and one on the acceptor DNA substrate (target DNA) which is biotinylated at its 5' end allowing it to interact with a streptavidin-europium fluorophore reagent. In LEDGF/p75-dependent integration assays,

the addition of cellular cofactor LEDGF/p75 is added to the reaction.

IN multimerization assays which measured the hypermultimerization of 6xHis and Flag-tagged INs in the presence of inhibitors were previously reported.<sup>25,41,48,55,73</sup> In brief, this assay monitors the interaction between two full-length wild-type HIV-1 IN proteins: one containing a 6xHis tag and the other a C-terminal FLAG tag. Anti-6xHis-XL665 and anti-FLAG-EuCryptate antibodies (Cisbio, Bedford, MA) allow an HTRF signal upon IN-IN interaction.

**HTS Data Availability.** The detailed experimental setup is available in the Supporting Information methods. The results are deposited in PubChem BioAssay AID 743277 (overall summary) with a total of 370,276 compounds tested in the primary LEDGF/p75-dependent integration assay. This resulted in a hit set of 2355 active hits (AID 743269). Follow-up counterscreen (AID 1053135, 2020 compounds tested), dose response curves (AID 1053172, 239 compounds tested with 164 active and 34 with activity below 1  $\mu\text{M}$ ), and confirmatory screens (AID 1053171, 239 compounds tested with 101 active and 15 with activity below 1  $\mu\text{M}$ ) were performed. Additionally, the same 2020 compounds were selected for the secondary screen of IN multimerization assay which resulted in 731 active hits (AID 1053136) and the corresponding counterscreen (AID 1053131 with 343 active). Of the 731 and 343 hits that were found to be active in the secondary and counterscreens, 291 were common in both.

**Antiviral Activity and Cytotoxicity Assay.** The antiviral activities ( $\text{EC}_{50}$ ) of the compounds with pNL4-3 WT replication competent viruses were determined in the full replication cycle as described previously.<sup>41,44</sup> The cytotoxicity assays ( $\text{CC}_{50}$ ) were performed as described previously.<sup>41</sup>

## ASSOCIATED CONTENT

### Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/acsomega.1c06378>.

Detailed methods of synthesis of all compounds; procedure for preparation of test compounds and general scheme for the HTS setup; HRMS,  $^1\text{H}$ - and  $^{13}\text{C}$  NMR chemical shifts for intermediates and compounds, 12, 14e, 15d, 15i, 15k, 16d, 16h, 16k, 22, 24a, 24b, 24c, 24d, 24e, 24f, 25a, 25b, 25c, 25d, 25e, 26a, 26b, 26c, 26d, 26e, 26f, 27, 28,  $^1\text{H}$ - and  $^{13}\text{C}$  NMR spectra for compounds 12, 13, 15d, 15i, 16d, 21a, 22a, 24a, 24b, 24c, 24d, 24f, 26f, and 27; commercial HTS compounds; compounds synthetized; HTRF curves against resistance mutants; and representative HTRF curves (PDF)

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## Author Contributions

D.A.-A., P.C.K., J.A., A.C.H., and J.K. performed the experiments and/or analyzed the experimental results. P.R.G., J.R.F., M.K., and R.C.L., designed and supervised separate sections of the study. R.C.L. together with M.K., J.K., J.R.F., and D.A.-A. conceived the entire study and wrote the manuscript with contributions from all other authors.

## Notes

The authors declare no competing financial interest.

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